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Journal of Insect Physiology 52 (2006) 1143–1152
Journal of Insect Physiology
www.elsevier.com/locate/jinsphys

Expression patterns of antibacterial genes in the Hessian fly

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Received 16 May 2006; received in revised form 25 August 2006; accepted 28 August 2006

Abstract

We report on the transcriptional patterns of three antibacterial genes, a defensin (*MdesDEF-1*), a diptericin (*MdesDIP-1*) and a lysozyme (*MdesLYS-1*), during development in Hessian fly, *Mayetiola destructor*. Quantitative analysis by real-time PCR of mRNA levels in different tissues revealed a predominance of the transcripts for all three genes in the midgut, while analysis during development revealed greatest abundance in mRNA during the 3rd-instar. An evaluation of the midgut lumen revealed the presence of a diverse bacterial flora in larvae maintained on susceptible wheat. Further, the titer of bacteria in the midgut increased ~250-fold from the 1st-instar through the 2nd-instar. However, no detectable titer of bacteria was observed from the midgut lumen of larvae maintained on resistant plants. PCR amplicons produced using primers designed to conserved regions of the *Pseudomonas* 16S rRNA gene supported taxonomic identification for some of the bacteria comprising the midgut flora as belonging to the genus *Pseudomonas*. Analysis of mRNA for the Hessian fly antibacterial genes in larvae feeding on susceptible and resistant plants revealed an increase in the transcript level for *MdesDEF-1* in 1st-instar larvae on susceptible plants, while the transcript levels for *MdesDIP-1* and *MdesLYS-1* were constant. Results suggest the transcriptional patterns of the Hessian fly antibacterial genes observed could be associated with the developing midgut bacterial flora present in larvae feeding on susceptible wheat as well as microbial challenge encountered at other stages in development.

Keywords: *Mayetiola*; Wheat; Antibacterial; Defensin; Diptericin; Lysozyme; Midgut; mRNA

1. Introduction

The Hessian fly, *Mayetiola destructor* (Say), is a major insect pest of bread and durum wheat, (*Triticum aestivum* L. and *Triticum turgidum* Desf.) in the United States and other parts of the world (Smiley et al., 2004). However, knowledge of the Hessian fly and its interactions with wheat at the molecular level is limited. Females deposit their eggs on the leaves of wheat plants and neonate larvae crawl down the leaves and feed near the crown (seedling wheat) or at nodes (jointing wheat). Damage to wheat is due entirely to feeding by larvae. On seedling wheat (fall infestation), larval feeding results in stunting and development of a dark green color in infested shoots or tillers and can lead to the death of seedling plants (Byers and Gallun, 1972). On jointing wheat (spring infestation), larval feeding prevents normal elongation of the stem and transport of nutrients to the developing grain (Buntin, 1999).

The most effective means of control for the Hessian fly is genetic resistance in the host plant (El Bouhssini et al., 2001), with 32 Hessian fly resistance genes identified so far (Sardesai et al., 2005a). This resistance is expressed as larval antibiosis and is controlled mostly by single genes that are partially or completely dominant (Gallun, 1977). Larvae infesting resistant wheat (an incompatible interaction) are unable to alter the plant's physiology, so that they can feed and develop normally, and die in 5–6 days

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0022-1910/$ - see front matter © 2006 Published by Elsevier Ltd.
doi:10.1016/j.jinsphys.2006.08.007

(Painter, 1930). In contrast, larvae maintained on susceptible wheat induce alterations in the plant that allow them to feed and develop normally (a compatible interaction).

The injury to susceptible wheat seedlings by feeding Hessian fly larvae has been reported to render the affected plant tissues vulnerable to attack by soil microorganisms (Boosalis, 1954). Further, the same study reported bacteria identified from within both feeding larvae and non-feeding 3rd-instar larvae present in puparia. Thus, Hessian fly larvae that have established a sustained feeding site on susceptible wheat are most likely subjected to microbial challenge even early in the 1st-instar.

In insects, an innate immune response via the production of antimicrobial peptides such as cecropins, attacins, drosomicins and defensins, is the primary line of defense against pathogens and parasites (Boman, 1998). Lysozymes are also an integral part of the defense mechanism against bacteria and fungi (Dunn, 1986; Fiolka et al., 2005) and were the first anti-microbial factors to be isolated from insect hemolymph (Powning and Davidson, 1976). Defensins, diptericins and lysozymes are part of the array of antimicrobial peptides responding to microbes, including gram-positive bacterial strains, gram-negative bacterial strains and fungi that can challenge insects (Imler and Bulet, 2005). The major site for production of these immune peptides is the fat body, from which they are secreted into the hemolymph (Hoffman, 1993; Imler and Bulet, 2005). However, synthesis of these immune peptides also occurs in other insect tissues/cells such as the midgut (Lehane et al., 1997; Li et al., 2005), salivary glands (Lamberty et al., 2001; Li et al., 2005; Klaudiny et al., 2005), Malphigian tubules (Li et al., 2005) and hemocytes (Lavine and Strand, 2002).

During analysis of expressed sequence tags (EST) from Hessian fly larval midgut cDNA libraries we recovered clones that encoded, respectively, a putative defensin, diptericin and lysozyme. The objectives of the present study were to: (1) reveal the spatial patterns of mRNA levels for the antibacterial genes in tissues and the temporal patterns during development; (2) evaluate larval midgut lumen content for bacterial flora present; (3) assess the antibacterial gene transcript levels in larvae feeding on susceptible and resistant wheat; (4) provide insight into the possible association of antibacterial proteins with microbial challenge in feeding larvae and at other stages in development.

2. Materials and methods

2.1. Insect and plant material

Hessian fly Biotype L maintained in culture as described by Sosa and Gallun (1973) was used in the present study. Biotype L was established from a field collection made from Posey County, Indiana in 1986. It is defined as able to survive on and stunt (virulent on) wheat lines carrying resistance genes *H3, H5, H6*, and *H7H8*, but is unable to survive on (avirulent on) the wheat line 'Iris' carrying resistance gene *H9*. The near isogenic wheat lines 'Newton' (carries no genes for resistance) and Iris (Patterson et al., 1994) were used in this study. Seedling plants at the one-leaf stage were mass-infested in chambers with Biotype L adults by covering the flats with a tent of nylon mesh and allowing females to oviposit on the seedling wheat. Larvae were then dissected at 1, 2, 3, and 4 days after hatch under a stereoscopic microscope using forceps. Biotype L larvae on Newton plants represented a compatible interaction (larvae on a susceptible wheat), while Biotype L larvae on Iris plants represented an incompatible interaction (larvae on a resistant wheat). Larvae removed from plants were collected in 1.5 ml micro-centrifuge tubes, flash-frozen in liquid nitrogen and stored at −80 °C until RNA was isolated.

2.2. Larval tissue dissection and RNA extraction

Approximately 300 midguts were dissected from late 1st- and early 2nd-instar larvae (Biotype L on Newton) immersed in ice-cold Schneider's insect medium (Sigma-Aldrich, St. Louis, MO). Midguts, salivary glands and fat body tissues were isolated as described by Mittapalli et al. (2005). All three tissues were collected in 100 μl of ice-cold Schneider's contained in a 1.5 ml micro-centrifuge tube. Immediately following collection, the tissues were flash-frozen in liquid nitrogen and stored at −80 °C until RNA was isolated. Total RNA from tissues, developmental stages (1st-, 2nd-, 3rd-instars, pupae and adults), and from larvae on susceptible Newton plants and on resistant Iris plants, was isolated using the RNAqueous®-4PCR kit from Ambion (Austin, TX).

2.3. Construction of midgut cDNA libraries

A cDNA library was constructed from the RNA extracted from the midgut tissue using a SMART™ cDNA library construction kit from BD Biosciences (Palo Alto, CA) following the manufacturer's protocol with one alteration; the cDNAs obtained were cloned directly into the PCR®4-TOPO® vector included in a TOPO TA cloning® for sequencing kit (Invitrogen, Carlsbad, CA) rather than cloning into the phage vector supplied with the BD Biosciences kit. Plasmid DNA was isolated using a Qiagen BioRobot 3000 (Valencia, CA) and cloned DNA EST fragments were sequenced from the 5′ end by the Purdue Genomics Center using a primer designed to the 5′ cloning oligonucleotide of the vector from the cDNA library construction kit.

2.4. Sequence comparison and secretion signal peptide analysis

Sequence similarity searches and annotations were done using BLAST programs on the National Center for Biotechnology Information (Bethesda, MD) website

(http://www.ncbi.nlm.nih.gov/). Analysis for secretion signal peptides was performed using SignalP v1.1 (Center for Biological Sequence Analysis, Technical University of Denmark, http://www.cbs.dtu.dk/services/SignalP/).

2.5. Quantitative analysis of mRNAs encoding Hessian fly antibacterial proteins

Levels of mRNA encoding the Hessian fly antibacterial proteins in various larval tissues were measured via quantitative real-time PCR (qRT-PCR). qRT-PCR was also used to assess transcript levels of *MdesDEF-1*, *MdesDIP-1* and *MdesLYS-1* during larval development on susceptible plants (compatible interaction) and on resistant plants (incompatible interaction). The primer sequences used for these analyses are referenced in Table 1. One microgram of total RNA in 10 μl of water was treated with DNase using the DNA-free kit (Ambion, Austin, TX) following the manufacturer's instructions. The reverse transcriptase reaction to generate the cDNA for use in q-RT-PCR was carried out using the SuperScript First Strand cDNA Synthesis kit (Invitrogen, Carlsbad, CA) as follows: 1 μl of oligo d(T) primer and 1 μl of dNTPs were added to the 10 μl of total RNA. The mixture was heated at 65 °C for 5 min and then placed on ice. The following were added on ice: 2 μl of 10 × first strand buffer, 2 μl of 50 mM $MgCl_2$, 2 μl of 0.1 M DTT, 1 μl of RNaseOut, and 1 μl of SuperScript II reverse transcriptase. cDNA synthesis was performed by at 42 °C for 2 h. Reactions were stopped by heating samples at 70 °C for 15 min.

The software Primer Express from Applied Biosystems (Foster city, CA) was used to design the real-time primers. Quantification of cDNA, displayed as relative expression value (REV) was based on the Relative Standard Curve method (User Bulletin #2: ABI Prism 7700 Sequence Detection System http://docs.appliedbiosystems.com/pebiodocs/04303859.pdf) using serial dilutions of a cDNA sample containing the target sequence. After qRT-PCR amplification the threshold cycle (Ct) value for each dilution was plotted against the log of its concentration, and Ct values for the experimental samples were plotted onto this dilution series standard curve. Target quantities were calculated from separate standard curves generated for each experiment. REVs were then determined by dividing the quantities of the target sequence of interest with the quantity obtained for ubiquitin. The entire analysis was performed using a Hessian fly ubiquitin as an internal standard, which in our evaluations has shown constant expression in the Hessian fly during development. Ubiquitin has been shown to be a suitable internal reference in a number of experimental systems (Jin et al., 2003; Luo et al., 2005; Yuan et al., 2006). PCR cycling parameters included 50 °C for 2 min, 95 °C for 10 min, and 40 cycles of 95 °C for 15 s, and 60 °C for 1 min.

2.6. Statistical analysis

For calculations of significance, the logs of the REVs for each gene were analyzed by ANOVA (Analysis of Variance) using the PROC MIXED procedure of SAS (SAS Institute Inc. SAS/STAT User's Guide, Version 9.1). For the expression analysis pertaining to tissues and developmental stages, the statistical model included treatment and interaction between treatments whereas for the analysis of expression in different larval Hessian fly/wheat interactions (compatible versus incompatible), the statistical model included treatment, time points, and interaction between treatments and time points as fixed effects. Biological replicates were included as a random effect in the analysis model. Treatment differences at each time point were evaluated using orthogonal contrasts and considered statistically significant if the p-value associated with the contrast was <0.05.

Relative fold-change in the case of tissue expression was calculated by taking the salivary gland mRNA experimental samples as the calibrator sample (confer User Bulletin #2: ABI Prism 7700 Sequence Detection System vide supra). Hence, the fold changes of the Hessian fly antibacterial gene transcripts in the midgut and fat bodies were calculated relative to the salivary glands. During development, the expression in the 1st-instars was taken as the calibrator. Fold change in gene expression of *MdesDEF-1*, *MdesDIP-1* and *MdesLYS-1* in larvae on susceptible plants versus virulent larvae on resistant plants was assessed by dividing the REV for larvae on susceptible plants by the REV for larvae on resistant plants for each of

Table 1
Primer sequences for quantitative real-time PCR

Gene	Primer sequence (5′-3′)	Tm (°C)	GenBank Accession no.
MdesDEF1-F	CTTACTCCAGACGCAGACAATGA	60.6	DQ017267
MdesDEF1-R	GGCACAAGCGGCTTGATTT	56.7	
MdesDIP1-F	TCGTGTGTATCAAAGCGATAACAA	57.6	DQ538392
MdesDIP1-R	TTCCATAGGGTCCACCAAGGT	59.8	
MdesLYS1-F	TGTGAAGATATGCTGGACGACAA	58.9	DQ538393
MdesLYS1-R	CCATGCAGTTTGAATATCATTTGAG	58.1	
MdesUBI1-F	CCCCTGCGAAAATTGATGA	54.5	DQ674274
MdesUBI1-R	AACCGGACTACTTGCATCGAA	57.9	

the four time points examined. For the tissue/developmental expression analysis two biological replicates were used, whereas, for expression analysis of larvae on susceptible versus on resistant plants three biological replicates were used. Further, two technical replicates were included in each biological replicate. The standard error represented the variance in these biological replicates for the respective analysis.

2.7. Profiling the midgut microflora

Midguts from 1st- and 2nd-instar larvae were dissected as described in Section 2.2. Lumen contents from ten 1st-instar midguts and two 2nd-instar midguts were extracted by pinching the midguts with sterile forceps and were collected in 100 μl of sterile water. Ten-fold serial dilutions to a final dilution factor of 1/10,000 and 25 μl of each dilution were spread on Difco™ potato dextrose agar (Sparks, MD) plates and incubated overnight at 37 °C under aerobic conditions. This was repeated in triplicate. Colonies were counted to determine the titer in colony forming units/midgut. Gram staining was performed for each type of colony with a distinct morphology using the Gram staining kit from Fluka Biochemika-Sigma-Aldrich Chemie GmbH (Buchs, Switzerland). The primary stain included in this kit was crystal violet and staining was done according to the manufacturer's protocol. Gram-positive bacteria stained bluish purple whereas, Gram-negative bacteria stained pinkish red. The observed characteristics for all the bacterial colonies are mentioned in Table 3.

2.8. PCR amplification of bacterial 16S rRNA genes

In order to reveal taxonomic information on midgut bacteria forming colonies, PCR was performed using primers designed to conserved regions of the 16S rRNA gene from *Pseudomonas* species (Widmer et al., 1998; Bennasar et al., 1996). These analyses included two sets of primers: Ps1F (5′-GGTCTGAGAGGATGATCAGT-3′), Ps1R (5′-TTAGCTCCACCTCGCGGC-3′), Ps2F (5′-AGAGTTTGATCMTGGCTCAG-3′), and Ps2R (5′-CGGTTACCTTGTTAGGACTTCACC-3′). The PCRs were performed as described by Bennasar et al. (1996). The PCR-amplified DNA fragments were analyzed on a 1% agarose gel followed by ethidium bromide staining. PCR amplicons were column purified using the Qiagen MiniElute PCR Purification kit according to manufacturer's protocol (Qiagen, Valencia, CA). PCR amplicons were then direct sequenced bi-directionally by the Purdue Genomics Facility.

3. Results

3.1. Characterization of the Hessian fly antibacterial genes

cDNAs containing complete open reading frames (ORFs) for three putative antibacterial genes, all of which encoded secretion signal peptides at their amino terminus, were recovered from the midgut EST project. The deduced amino acid sequences for all three putative Hessian fly antibacterial genes were in agreement with similar sequences from other insects. MdesDEF-1 showed 59% similarity (6e−10; identities plus conserved substitutions) at the amino acid level with a defensin from *Aedes aegypti* (AAD40115); MdesDIP-1 showed 55% similarity (5e−13) with a diptericin from *Drosophila melanogaster* (AAB82531); and MdesLYS-1 showed 63% similarity (2e−33) with a lysozyme from *Bombyx mori* (AAB40947). The nucleotide and deduced amino acid sequences for *MdesDEF-1, MdesDIP-1* and *MdesLYS-1* were submitted to GenBank and were assigned the accession numbers DQ017267, DQ538392, and DQ538393, respectively. Further, key amino acid residues such as cysteines were also found to be conserved among the derived Hessian fly sequences.

3.2. Expression analyses of the Hessian fly antibacterial genes in larval tissues

Quantitative real-time PCR (qRT-PCR) was used to assay the temporal patterns of mRNA from *MdesDEF-1*, *MdesDIP-1*and *MdesLYS-1* in different larval tissues, including midgut, salivary glands and fat bodies of virulent late 1st-instars. Fig. 1 shows that all three mRNAs were present in the midgut tissue and the most abundant of the three was *MdesDEF-1*. The lowest level observed for the three Hessian fly antibacterial genes was in the salivary glands and the mRNA levels in the midgut and fat bodies were compared relative to this basal level in salivary glands. A 15.7-fold ($p<0.05$) change was calculated between the midgut and salivary tissues for *MdesDEF-1*, while a 7.1- and 8.4-fold ($p<0.05$) change was revealed for *MdesDIP-1* and *MdesLYS-1* between the same tissues.

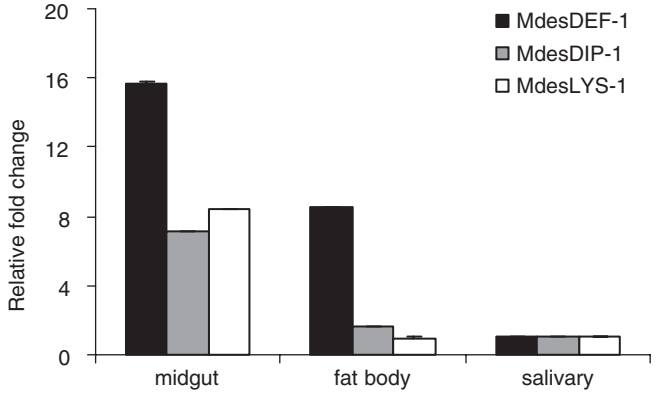


Fig. 1. mRNA levels for antibacterial genes in Hessian fly larval tissues. Quantitative analysis determined levels of mRNAs encoding Hessian fly antibacterial proteins in 1st-instar larval midgut, fat body and salivary glands. The salivary gland sample was taken as the calibrator (assigned a value of 1) and the mRNA levels in midgut and fat body samples were calculated relative to the levels in the salivary glands. The standard error of the mean for two biological replicates is represented by the error bar.

The difference in mRNA levels between the midgut and fat bodies for *MdesDEF-1*, *MdesDIP-1* and *MdesLYS-1* was 1.8-, 4.4- and 8.8-fold ($p = 0.05$, 0.05 and <0.05), respectively (Fig. 1).

3.3. Quantification of mRNAs encoding the Hessian fly antibacterial genes during development

Quantitative analysis was also performed to assay the temporal patterns of mRNA for *MdesDEF-1*, *MdesDIP-1*and *MdesLYS-1* in different stages of development including the 1st-, 2nd-, and 3rd-instars, pupae and adults. qRT-PCR data suggested that the greatest level of mRNA for all three genes was in the 3rd-instars (Fig. 2A). The lowest level of transcript detected for all three Hessian fly antibacterial genes was in the 1st-instars and therefore, relative levels during development were assessed by comparing the mRNA levels in other stages to this basal level in 1st-instar larvae. For *MdesDEF-1*, a 5-fold ($p<0.05$) increase in mRNA was revealed between the 3rd- and 1st-instars and a 4-fold ($p<0.05$) between the 2nd- and 1st-instars (Fig. 2B). For *MdesDIP-1*, a 6-fold ($p<0.05$) increase in mRNA was revealed between the 3rd- and 1st-instars and a 2-fold ($p = 0.05$) between the 2nd- and 1st-instars. A 5.9-fold ($p<0.05$) change between the 3rd- and 1st-instars and a 2.3-fold ($p = 0.05$) change between the pupae and 1st-instars was revealed for *MdesLYS-1* (Fig. 2B).

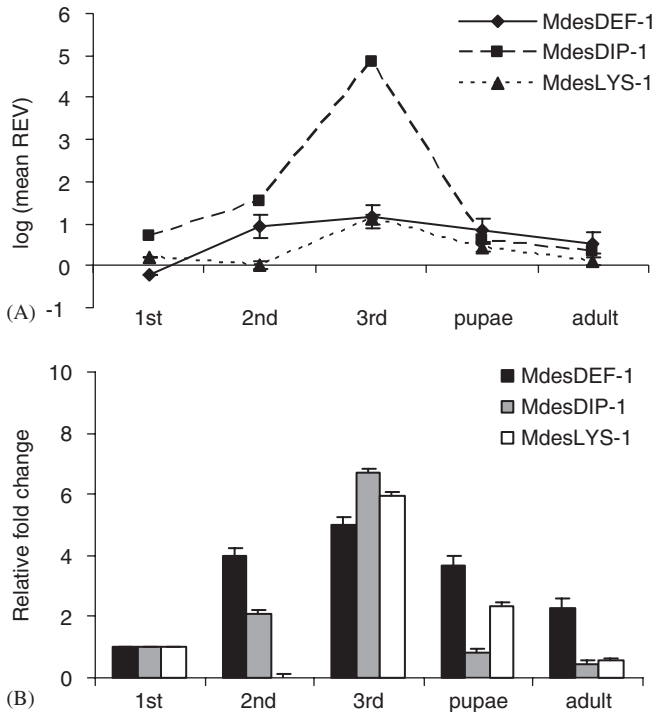


Fig. 2. mRNA levels for antibacterial genes during Hessian fly development. (A) Quantitative analysis of mRNA encoding Hessian fly antibacterial proteins in different stages of development including 1st-, 2nd-, 3rd-instars, pupae and adults. Log of the mean relative expression value (REV) is plotted against each of the five developmental stages examined. (B) Relative fold change of the Hessian fly antibacterial mRNAs during development. The 1st-instar sample was taken as the calibrator (assigned a value of 1) and the mRNA levels in other stages were calculated relative to the levels in the 1st-instars. In both panels, the standard error for two biological replicates is represented by the error bar.

3.4. Temporal and spatial expression patterns of the Hessian fly antibacterial mRNAs during compatible and incompatible interactions with wheat

qRT-PCR was used to estimate the REVs for developing 1st-instar Biotype L larvae on susceptible Newton plants (compatible interaction) and on the resistant Iris plants (incompatible interaction). *MdesDEF-1*, mRNA levels were significantly greater ($p<0.05$) in larvae during the compatible interaction from 2 days after egg hatch onwards (Fig. 3A). This was reflected in the 5.4-, 3.9-, and 6.3-fold ($p<0.05$) increase for 2-, 3-, and 4-day post-hatch larvae in the compatible interaction compared to similar aged larvae in the incompatible interaction. Data for *MdesDIP-1*, revealed a significant increase ($p<0.05$, 4.2-fold) in mRNA levels for only 2-day post-hatch larvae in the compatible interaction compared to similar aged larvae in the incompatible interaction (Fig. 3B). For *MdesLYS-1*, there was no significant difference ($p>0.05$) in levels of the antibacterial mRNAs at any of the four time points examined between larvae in compatible and incompatible interactions with wheat (Fig. 3C).

3.5. Determining the presence and titer of midgut microflora in the 1st- and 2nd-instars of the Hessian fly

Preliminary analysis of the midgut lumen of virulent and avirulent 1st-instars and the green-gut stage (feeding 2nd-instar) of virulent Biotype L Hessian fly larvae suggested the presence of a diverse microflora inhabiting the midgut. Several strains of bacteria were observed, which were classified as Gram-positive or Gram-negative rods or cocci. Gram-positive bacteria stained bluish purple and Gram-negative bacteria stained pinkish red. It was found that the majority of the bacteria identified in both instars fell within the class categorized as Gram-negative rods (Fig. 4). There were at least eight different colony types identified in the 1st-instar (Fig. 4A) of which only four are shown in Fig. 4B(1–4). Three of the four colony types were classified as Gram-negative rods (Fig. 4B-1, 2, 4) and one as Gram-positive cocci. However, in the green-gut stage (Fig. 4C), colony diversity was restricted to two major types, with the predominant colonies being white (Fig. 4D-2). This analysis also revealed the titer (colony forming units/midgut) to increase ~250-fold from 1st -instars to the green-gut stage (Table 2) However, no detectable titer of bacteria was observed from the midgut lumen of 1st-instar avirulent larvae (Table 2). (Table 3)

The PCR analysis using primers designed to conserved regions of the *Pseudomonas* 16S rRNA gene and sequence analysis of the resulting amplicons suggested the genus for the bacteria from midgut lumen contents forming yellow

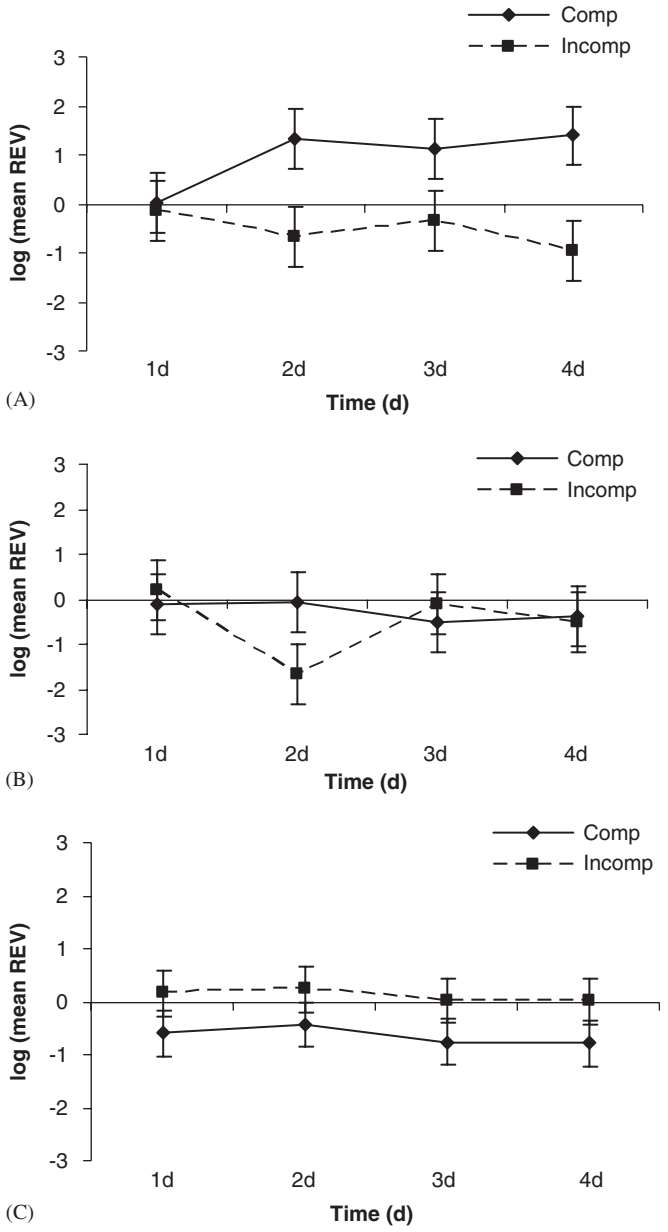


Fig. 3. Temporal patterns of mRNA encoding Hessian antibacterial proteins. Samples of virulent (compatible interaction) and avirulent (incompatible interaction) Hessian fly larvae were collected through the 1st-instar (1–4 days post hatch). Profiles for (A) *MdesDEF-1*, (B) *MdesDIP*-1 and (C) *MdesLYS*-1 are shown. Log of the mean relative expression value (REV) is plotted against each of the four time points examined. The standard error for three biological replicates is represented by the error bar.

colonies to be *Pseudomonas* (Figs. 4B-4 and 4D-1). However, for all the other bacterial colonies there were no PCR-derived products detected (data not shown).

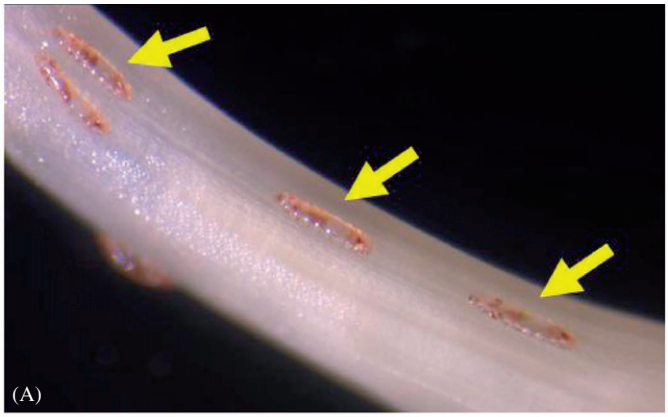
4. Discussion

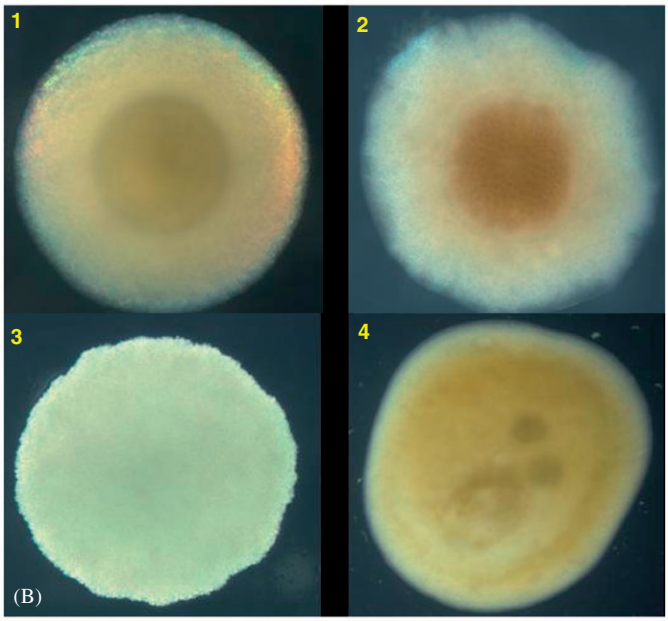
Annotation of cDNAs from an EST project that focused on genes expressed in the midgut of feeding Hessian fly larvae has identified three putative antibacterial genes: a defensin (*MdesDEF-1*), a diptericin (*MdesDIP-1*) and a lysozyme (*MdesLYS-1*). A diverse set of antibacterial genes in addition to the ones identified in the Hessian fly have been reported in the genome of *Drosophila melanogaster* including cecropins, attacins and drosocin (Dimarcq et al., 1994; Hedengren et al., 2000; Imler and Bulet, 2005).

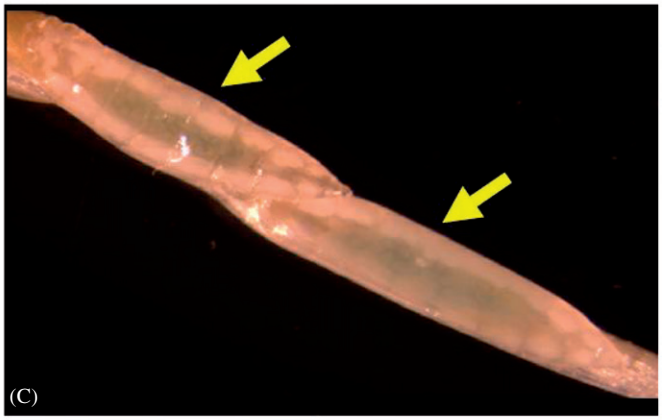
Following infection or septic injury, insect antimicrobial peptides are rapidly synthesized, are cleaved by signal peptidases, become functionally active, and are then rapidly secreted into the hemolymph (Imler and Bulet, 2005). Once in the hemolymph they target harmful microbes to defend the host (Boman, 1998; Bulet et al., 2004; Lamberty et al., 2001). Evidence that proteins, encoded by the Hessian fly antibacterial genes reported in the current study, are secreted is the presence of a secretion signal peptide sequence at the amino-terminus in the deduced amino acid sequences. These results support the hypothesis that these Hessian fly antibacterial proteins could be transported into the hemolymph and/or to the lumen of the midgut protecting the insect from micro-organisms.

Results from temporal tissue expression analysis performed in the current study revealed that all three Hessian fly antibacterial genes were expressed prominently in the midgut but also at considerable levels in the fat body, and at low levels in the salivary glands (Fig. 3). Similar results have been reported in other insect species where synthesis of antibacterial mRNA occurs in fat body and midgut of the blood-sucking bug, *Rhodnius prolixus* (Lopez et al., 2003) and in the greater wax moth, *Galleria mellonella* (Lee et al., 2004). In *D. melanogaster* the major tissue producing these antibacterial proteins is fat body (Hoffman, 1993); however, they are also expressed in other tissues in a tissue-specific manner (Imler and Bulet, 2005). More recently, some of the c-type lysozymes in the African malaria mosquito, *Anopheles gambiae* were observed to be expressed in several tissues including midgut, Malphigian tubules and salivary glands (Li et al., 2005).

The role of antimicrobial peptide genes specifically expressed in the midgut of many insects has also been investigated but to a lesser extent than those expressed by fat body and hemocytes. Some of the best-studied midgut-specific defensins in insects are from blood-feeding arthropods such as *A. gambiae* (Dimopoulos et al., 1997), the stable fly, *Stomoxys calcitrans* (Lehane et al., 1997; Munks et al., 2001), as well as the arachnid soft tick, *Ornithodoros moubata* (Nakajima et al., 2001). Two defensin genes, *Smd1* and *Smd2*, cloned from the anterior midgut tissue of *S. calcitrans* were found to be important in conferring protection against bacterial invasion in the midgut epithelial tissue and not in the hemolymph (Lehane et al., 1997). Further, in the soft tick *O. moubata*, four isoforms of defensins possessing antimicrobial properties were identified, of which three isoforms were specific in their expression to the midgut, while the fourth was primarily expressed in the fat body (Nakajima et al., 2001). High levels of two c-type lysozymes (Lys c-1 and Lys c-7)







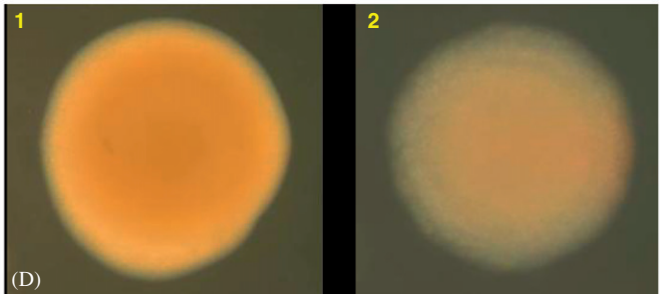


Table 2
Diversity and titer of bacterial colonies in the midgut of Hessian fly larvae

Larval instar (days after hatch)	Diversity (no. colony types)	Colony-forming units (cfu) per midgut
Virulent larvae[a]		
1st-instar (3 days)	8	~3
2nd-instar (10 days)	2	~750
Avirulent larvae[b]		
1st-instar (3 days)	0	0

[a]Biotype L larvae on susceptible 'Newton' wheat.
[b]Biotype L larvae on resistant 'Iris' wheat.

were also observed in the midgut tissue of *A. gambiae* post blood feeding (Li et al., 2005).

Salivary gland secretions play an important role in the initial interactions between Hessian fly larvae and their host plant. Even the low level of *MdesDEF-1* expression in the salivary gland could protect the epithelium from microbes that the larvae may encounter while feeding and/or due to septic injury resulting from abrasion of the feeding surface. In the honeybee (*Apis mellifera*) it was observed that defensin-1 and defensin-2 were expressed in thoracic tissues suggesting their presence in the saliva (Klaudiny et al., 2005). The primary functions of these defensins in the honeybee saliva were speculated to be protection of the salivary epithelial tissue and disinfection of the surface of wax honeycomb cells inside the beehive. Similarly, in the fungus-growing termite *Pseudocanthotermes spiniger* a cysteine-rich antifungal peptide known as 'termicin' was found to be expressed constitutively in the salivary glands (Lamberty et al., 2001). A suggested role for termicin in the salivary glands of *P. spiniger* was protection of eggs against fungi since the eggs are coated with saliva by workers during development.

During development all three Hessian fly antibacterial genes had varying transcript patterns. *MdesDEF-1* showed the highest level of mRNA in feeding 2nd-instars and non-feeding 3rd-instars. In contrast, *MdesDIP-1* and *MdesLYS-1* transcripts were most abundant in the 3rd-instars and pupae. Larvae of both *A. gambiae* and *D. melanogaster* have been reported to have high levels of antimicrobial mRNA, which most likely are involved in response to bacterial challenge encountered during larval

Fig. 4. Bacterial colonies obtained with extract of larval Hessian fly midgut lumen. (A) 1st-instar Hessian fly larvae 3 days post-hatch (arrows) feeding at the crown portion of wheat. (B) Bacterial colonies recovered with midgut extract from 1st-instar Hessian fly larvae after an overnight incubation under aerobic conditions. The four prominent types of bacterial colonies (1–4) observed from the lumen content extracted from 3-day-old 1st-instar Hessian fly larvae are shown. (C) 2nd-instar green-gut larvae 10 days post-hatch (arrows) removed from the crown portion of a wheat plant. (D) Bacterial colonies recovered with midgut extract from green-gut 2nd-instar larvae after an overnight incubation under aerobic conditions. The two types of bacterial colonies (1–2) observed from the midgut lumen of green-gut larvae are shown. All images are presented at a total magnification of 20 ×.

Table 3
Characterization of bacteria and colonies formed

Colony no.	Gram reaction	Form of bacteria	Colony morphology (shape and color)	Putative ID (genus)
B1	Gram-negative	Rods	Round, yellow center	Unknown
B2	Gram-negative	Rods	Irregular, yellow center	Unknown
B3	Gram-negative	Cocci	Irregular, white	Unknown
B4	Gram-positive	Rods	Irregular, yellow	*Pseudomonas*
D1	Gram-negative	Rods	Irregular, yellow	*Pseudomonas*
D2	Gram-negative	Rods	Round, white	Unknown

development (Daffre et al., 1994; Li et al., 2005). However, activity of such anti-bacterial factors also has been thought to be of importance in other stages of development. For example, expression of three of the eight c-type lysozymes (Lys c-1, Lys c-6 and Lys-c-7) in *A. gambiae* were relatively abundant in the later stages of development including pupae and adults. The high mRNA levels observed in the present study for *MdesDEF-1*, *MdesDIP-1* and *MdesLys-1* in the non-feeding Hessian fly 3rd-instars and pupae could be related to microbial challenge during these stages in development. The low levels of antibacterial mRNA in avirulent 1st-instar larvae may be due to lack of microbe consumption, since these larvae may encounter plant substances that serve as feeding deterrents (Subramanyam et al., 2006).

We have shown the presence of a diverse range of bacteria in the midgut of both 1st- and 2nd-instars of virulent Hessian fly larvae (Fig. 4). A previous study by Boosalis (1954) reported the presence of various microorganisms in the Hessian fly including bacteria and fungi. The current study found a greater diversity in bacterial colonies recovered from the midguts of feeding larvae compared to bacteria obtained from internal portions of the pupae (Boosalis, 1954). The two major types of bacterial colonies identified by Boosalis (1954) were described as being either white or yellow. In our analysis we observed a large variation both in the diversity of bacterial colonies and in the titer within 1st- and 2nd-larval instars (Table 2). All the bacterial colonies described as yellow were positive for the PCR-based assay using primers targeting the 16S rRNA genes from the genus *Pseudomonas*. However, the negative results obtained with the other colony types does not preclude them from being *Pseudomonas* as only two sets of conserved primers were used in this study. Additionally, the negative colonies could be other gram-negative bacteria such as members of the family Enterobacteriaceae. These results along with the differential patterns of the Hessian fly antibacterial gene transcripts in tissues and during development could imply a role for the encoded proteins in the modulation of the midgut microflora. Further, *MdesDEF-1*, *MdesDIP-1* and *MdesLys-1* may function in protecting the insect from microbes of various origins and limit the diversity of such species (i.e., a decrease in the diversity of bacterial colony types recovered from midgut lumen contents in the 2nd-instar compared to the 1st-instar).

The lack of a detectable titer of bacteria in the midgut lumen of avirulent larvae (Table 2) could be due to two plausible reasons. First, avirulent larvae fail to establish a sustained feeding site in wheat seedlings (Shukle et al., 1990). This could preclude the ingestion of microorganisms and the development of a gut microflora by avirulent larvae. Second, there are several lines of defense, which these larvae encounter when they attack resistant plants that could also kill bacteria. It has been reported that the mRNA level for a lipoxygenase (*WCI-2*) in resistant wheat plants becomes more abundant in resistant plants upon infestation by avirulent Hessian fly larvae (Sardesai et al., 2005b). Lipoxygenases produce reactive oxygen species (ROS) including lipid hydroperoxides and hydrogen peroxide (Kanofsky and Axelrod, 1986). However, other ROS could also be generated within the larvae due to stress (Mittapalli et al., unpublished data). Thus, ROS could be responsible in sterilizing the midgut of larvae infesting resistant wheat plants.

The temporal patterns of expression for all three Hessian fly antibacterial genes in virulent and avirulent 1st-instar larvae suggested *MdesDEF-1* to have a protective role during larval feeding. *MdesDEF-1* may be involved in protecting larvae against microbial invasion during feeding on wheat seedlings. However, in the case of *MdesDIP-1* and *MdesLYS-1*, there was no indication of the levels of transcripts for these genes increased during the initial phase of larval interaction and feeding on the host plant. This observation was further supported by transcriptional profiles in tissues and during development, for which *MdesDEF-1* seemed to be the most abundant transcript of the three Hessian fly antibacterial genes assayed. Defensins have been shown to be active against an array of microbes including gram-positive and gram-negative bacteria and also filamentous fungi (Imler and Bulet, 2005). On the other hand, diptericins are effective against gram-negative bacterial strains (Dimarcq et al., 1988; Lambert et al., 1989; Imler and Bulet, 2005) and lysozymes against gram-positive bacterial strains (Schneider, 1985; Imler and Bulet, 2005). Except for one colony type, all the other colonies screened in this study were identified as containing gram-negative bacterial strains. In *D. melanogaster* the expression of *diptericin* was suppressed with a high bacterial (gram-negative strains) infection dose (Lindmark et al., 2001) and growth rate (Johansson et al., 2006). In a similar manner, the antibacterial action of *MdesDIP-1* could be

negatively affected with the high dose and diversity of gram-negative bacteria present in the midgut of larvae feeding on wheat seedlings.

In the present study we have recovered from Hessian fly cDNAs containing complete open reading frames (ORFs) encoding three putative antibacterial proteins, all of which contained secretion signal peptides at their amino termini. Expression analysis revealed the transcripts levels were highest in the larval midgut and at low levels in the salivary glands and fat body. Transcripts for the antibacterial genes were also expressed in all stages of development including the three larval instars, pupae and adults, suggesting a role in response to bacteria encountered by Hessian fly feeding on wheat and during development. We speculate on the possible functions of the Hessian fly antimicrobial genes based on their expression patterns in virulent larvae and the absence of a bacterial flora in the midgut of avirulent larvae. However, further studies of these putative antibacterial genes will be required to reveal their precise functions in the biology of the Hessian fly.

Acknowledgements

We thank the anonymous reviewers for their critical suggestions. We acknowledge Dr. Martin Gonzalo (Department of Agronomy, Purdue University) for assistance with the statistical analysis. Technical support provided by John Shukle is greatly appreciated. This is a joint contribution of the USDA-ARS and Purdue University. Purdue University Agricultural Experiment Journal Paper 2005-17679. This article represents the results of research only. Mention of a commercial or proprietary product does not constitute an endorsement by the USDA for its use. This study was supported through USDA CRIS No. 3602-22000-014D.

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